with 20 ml of the ice-cold washing buffer (50 mM Tris/HCl [pH 6.3] containing 50 mM NaCl). The cell pellet was resuspended in an ice-cold Hepes buffer (100 mM Hepes [pH 7.5] containing 20% Glycerol) to reach a final concentration of 25 g dry cell weight/liter. Resuspended cells were permeabilized by adding 30 µl of a 10% Hexadecyltrimethyl-ammonium bromide (CTAB) (w/v) solution to 1 ml of cells to give a final concentration of 0.3% (CTAB)(v/v).

[0055] For determination of pyruvate carboxylate activity, the assay mixture contained 10 mM pyruvic acid, 14 mM $KHCO_3$, 4 mM $MgCl_2$, 1.75 mM ATP, 50 µmole acetyl-CoA, 0.3 mg bovine serum albumin, 0.055 U citrate synthase and 50 mM sodium phosphate buffer ([pH 7.5] containing 0.1 mg 5, 5′-Dithio-bis(2-nitrobenzoic acid) (DTNB)) in a final volume of 1 ml. The reaction was started at 30°C with the addition of 10 µl of the permeabilized cell suspension, and the formation of DTNB-thiophenolate was followed over time at 412 nm. Relevant standards and controls were carried out in the same manner.

[0056] For determination of phosphoenol pyruvate carboxylase activity, the assay mixture contained 10 mM phosphoenol pyruvate, 14 mM $KHCO_3$, 4 mM $MgCl_2$, 50 µmole acetyl-CoA, 0.3 mg bovine serum albumin, 0.055 U citrate synthase and 50 mM sodium phosphate buffer ([pH 7.5] containing 0.1 mg 5, 5′-Dithio-bis(2-nitrobenzoic acid) (DTNB)) in a final volume of 1 ml. The reaction was carried out in the same conditions described for the pyruvate carboxylase assay.

[0057] The reproducibility for enzyme assays was typically 10%.

DNA Isolation and Purification

[0058] DNA was isolated from cultures of NRRL B-11474 cells. Defined media for NRRL B-11474 (CM media) contain the following ingredients, per liter: sucrose, 50 g; KH_2PO_4, 0.5 g; K_2HPO_4, 1.5 g; urea, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; polypeptone, 20 g; beef extract, 5 g; biotin, 12.5 ml (60 mg/L); thiamine, 25 ml (120 mg/L), niacinamide, 25 ml (5g/L); L-methionine, 0.5 g; L-threonine, 0.25 g;

L-alanine, 0.5 g. NRRL B-11474 cells were harvested from CM media and suspended in 10 ml of TE, pH 8 (10 mM Tris*Cl, 1 mM EDTA). Forty micrograms of RNase A and 10 milligrams of lysozyme were added per milliliter of suspension and the suspension was incubated at 37°C for 30 minutes. The suspension was made in 1.0% in sodiumdodecyl sulfate (SDS) and 0.1 mg/l proteinase K was added, and the cells were lysed by incubation at 37°C for 10 minutes. Nucleic acids were purified by three extractions with TE-saturated phenol (pH7), followed by ethanol precipitation. Nucleic acid precipitates were twice washed with 80% ethanol and redissolved in TE pH 8.

[0059] The concentrations of DNA were quantified spectrophotometrically at 260 nm. Purity of DNA preparations were determined spectrophotometrically (A260/A280 and A26JA230 ratios) and by agarose gel electrophoresis (0.8% agarose in 1x TAE).

[0060] Sequencing of the genomic DNA was performed, as is known by one of ordinary skill in the art, by creating libraries of plasmids and cosmids using pGEM3 and Lorist 6 respectively. Briefly, a Sau3AI digestion was performed on the genomic DNA and inserted into the BamHI site of pGEM3. The forward primer was used to generate a sequence, and primer walking generated the remainder of the sequence.

Activity of Pyruvate Carboxylase

Development of a Continuous Assay for Determining Pyruvate Carboxylase Activity

[0061] A discontinuous assay for determining pyruvate carboxylase from permeabilized cells has been previously described (Peters-Wendisch, P.G. *et al. Microbiology,* 143: 1095-1103 (1997)). Because of the central location of OAA in the metabolism, it seemed to be that OAA would accumulate during the first reaction of the discontinuous assay. Most likely, OAA would be lost to other

products, because of the competing enzymes that are still active. This depletion of OAA would inevitably lead to the underestimation of pyruvate carboxylase activity. To verify this assumption of decreasing OAA concentrations, a known amount of OAA was added to the first reaction in presence of permeabilized and non-permeabilized cells. A significant loss of OAA was detected, demonstrating that permeabilized cells are capable of further transformation of OAA.

[0062] To account for the intrinsic loss of OAA during the experiment, a continuous assay was carried out by coupling the two-reaction assay to a one-reaction assay in presence of an excess of citrate synthase. The amount of permeabilized cells added in the assay was optimized to obtain a detectable activity, with the lowest possible background absorbency due to the presence of cells.

[0063] To confirm that the continuous assay specifically detected pyruvate carboxylase activity, controls were carried out by assaying for activity in absence of each reaction component (Table 1). Using these controls, the detected activity was determined to be a carboxylation reaction requiring pyruvate, Mg and ATP.

Table 1: Controls for the continuous pyruvate carboxylate assay.

Control	Detected Activity (Abs/min.mg DCW)
Complete mixture	0.30
Cells omitted	0
Pyruvate omitted	0.01
$KHCO_3$ omitted	0.03
$MgCl_2$ omitted	0.02
ATP omitted	0.03
Citrate synthase omitted	0.10
Complete + biotin	0.35
Complete + avidin	Not determined yet

To optimize the assay, the influence of the ratio of CTAB:cells was tested. Maximal activity was measured between 8 and 24 mg CTAB/mg dry cell weight (DCW). Pyruvate carboxylase activity was measured in cells incubated with